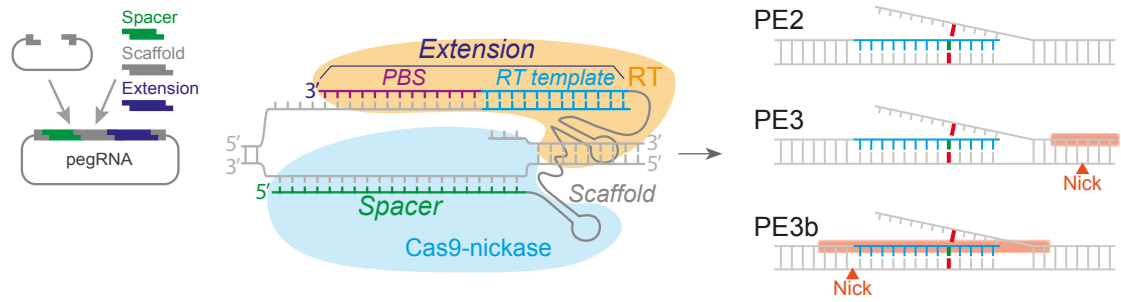
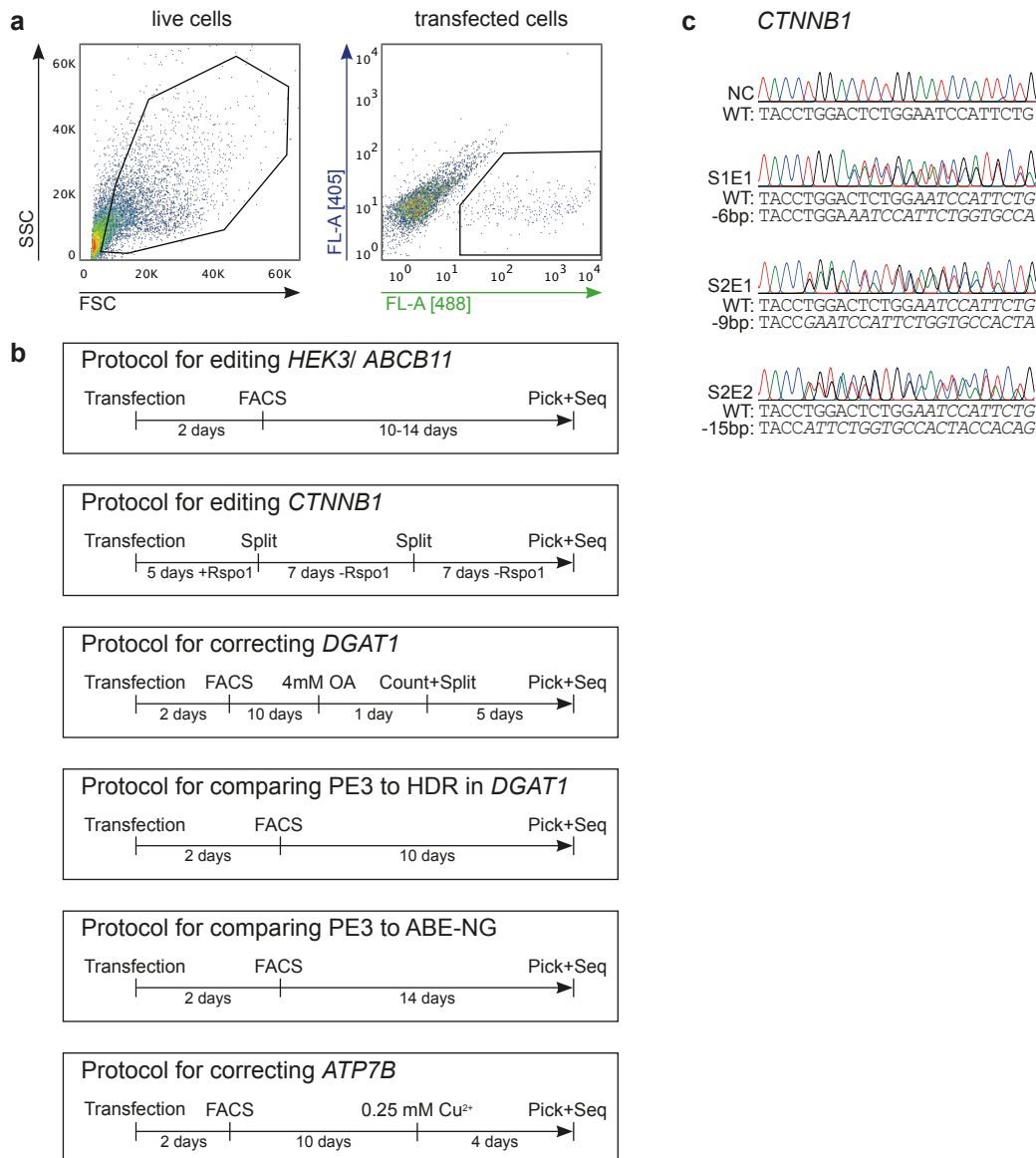




### Supplementary Figure 1

#### Overview of the prime editing principle

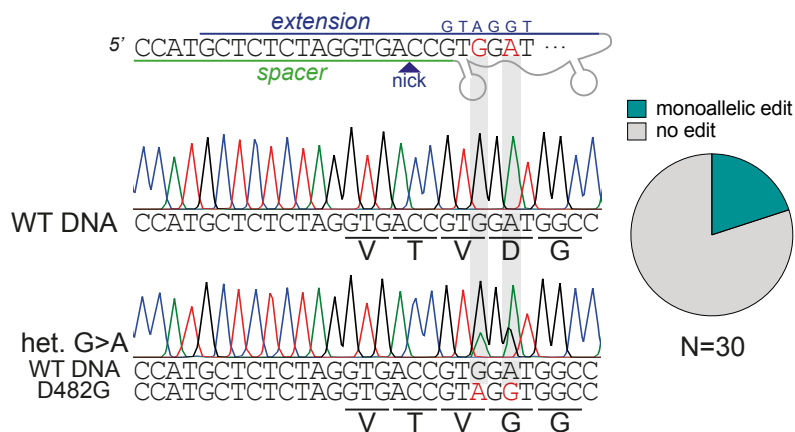
Schematic representation of the pegRNA structure and the mechanism by which a pegRNA molecule binds to genomic DNA to instruct PE2 (Cas9-reverse transcriptase fusion complex) to bind to a nick a specific sequence, and synthesize a new, edited flap according to the RT template. The PE3 strategy adds a nick in the non-edited strand to enhance incorporation of the edited flap. The PE3b strategy is similar to PE3, but only nicks the opposite strand after successful creation of an edited flap. PBS: primer binding site, RT: reverse transcriptase.



## Supplementary Figure 2

### Representative FACS plot example and different protocols used in this study

**a**, Representative FACS plot and gating strategy to sort transfected cells. Sorting of transfected cells was based on co-transfection with a GFP-reporter plasmid in all transfection experiments. **b**, Workflow and timeline for transfection of organoid cells, selection of single transfected cells, and characterization of prime editing efficiency in clonally expanded cells. Note that the workflow used for prime editing of *CTNNB1* does not contain FACS-based selection of transfected cells, but instead selects for *CTNNB1* mutants based on withdrawal of R-spondin 1 (Rspo1). **c**, Sanger validation of precise 6-bp, 9-bp, and 15-bp deletions in picked clones from *CTNNB1* pegRNA S1E1, S2E1, and S2E2, respectively. SSC: side scatter, FSC: forward scatter, OA: oleic acid.



### Supplementary Figure 3

#### Generation of *ABCB11*<sup>D482G</sup> by prime editing (PE3)

Design of pegRNA and Sanger validation of generation of the pathogenic *ABCB11*<sup>D482G</sup> mutation in liver organoids. Note that *ABCB11*<sup>D482G</sup> is created by an A→G substitution at position 7, but the pegRNA additionally introduces a silent A→G mutation at position 5, disturbing the PAM sequence to increase efficiency.

HEK3 unwanted byproduct analysis

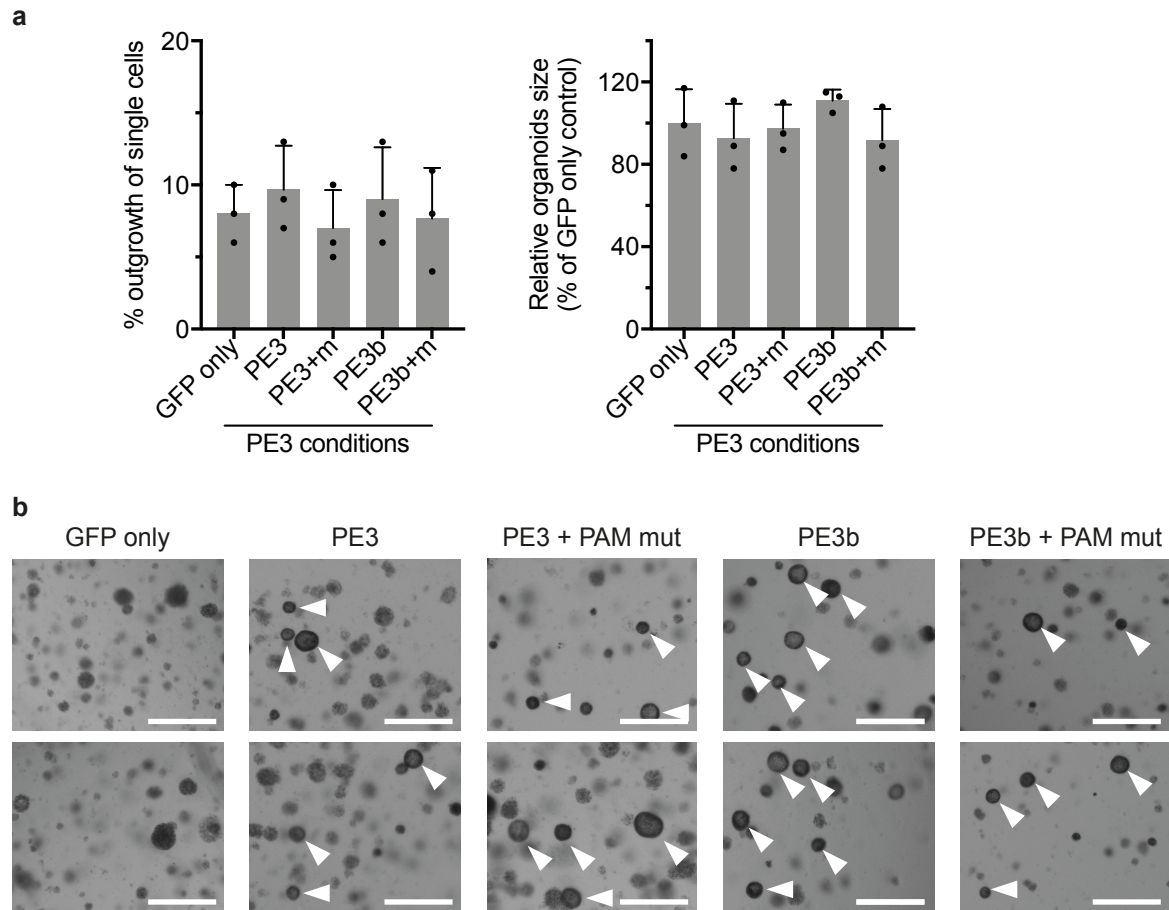
| Pattern | %      | Reads | Cell type  |
|---------|--------|-------|------------|
| WT      | Fig 1h | -     | -          |
| 5bp del | Fig 1h | -     | -          |
| G>A +9  | 2.392  | 1198  | intestinal |
| G>A +9  | 1.022  | 95    | liver      |
| G>A +9  | 0.898  | 114   | intestinal |
| G>A +9  | 0.864  | 216   | intestinal |
| G>A +9  | 0.685  | 217   | HEK293T    |
| G>A +9  | 0.682  | 295   | HEK293T    |
| G>A +9  | 0.534  | 311   | HEK293T    |
| G>A +9  | 0.271  | 73    | liver      |
| C>A +23 | 1.549  | 491   | HEK293T    |
| C>A +23 | 0.78   | 200   | Caco-2     |
| C>A +23 | 0.737  | 98    | liver      |
| C>A +23 | 0.528  | 132   | intestinal |
| C>A +31 | 1.495  | 474   | HEK293T    |
| C>A +31 | 0.925  | 123   | liver      |
| C>A +31 | 0.74   | 185   | intestinal |
| C>A +31 | 0.709  | 182   | Caco-2     |
| C>A +31 | 0.25   | 108   | HEK293T    |
| C>A -9  | 0.776  | 246   | HEK293T    |
| C>A -9  | 0.489  | 65    | liver      |
| C>A -9  | 0.444  | 114   | Caco-2     |
| C>A -9  | 0.412  | 103   | intestinal |
| G>A +9  | 0.669  | 89    | liver      |
| G>A +9  | 0.446  | 193   | HEK293T    |
| G>A +9  | 0.444  | 111   | intestinal |
| G>A +9  | 0.42   | 39    | liver      |
| G>A +9  | 0.302  | 176   | HEK293T    |
| G>A +9  | 0.28   | 140   | intestinal |
| C>A +23 | 0.656  | 208   | HEK293T    |
| C>A +23 | 0.632  | 84    | liver      |
| C>A +23 | 0.394  | 101   | Caco-2     |
| C>A +23 | 0.328  | 82    | intestinal |
| G>T +12 | 0.609  | 193   | HEK293T    |
| G>T +12 | 0.448  | 115   | Caco-2     |
| G>T +12 | 0.391  | 52    | liver      |
| G>T +12 | 0.364  | 91    | intestinal |
| C>A +30 | 0.606  | 192   | HEK293T    |
| C>A +30 | 0.399  | 53    | liver      |
| C>A +30 | 0.277  | 71    | Caco-2     |

CTNNB1 unwanted byproduct analysis

| Pattern | %      | Reads | Cell type  |
|---------|--------|-------|------------|
| WT      | Fig 1h | -     | -          |
| 6bp del | Fig 1h | -     | -          |
| G>A +6  | 1.894  | 2830  | intestinal |
| G>A +6  | 1.036  | 732   | HEK293T    |
| G>A +6  | 0.947  | 387   | HEK293T    |
| G>A +6  | 0.76   | 509   | liver      |
| G>A +6  | 0.759  | 351   | HEK293T    |
| G>A +6  | 0.757  | 1007  | intestinal |
| G>A +6  | 0.588  | 497   | intestinal |
| G>A +6  | 0.486  | 409   | liver      |
| G>A +6  | 0.454  | 322   | Caco-2     |
| G>A +6  | 0.379  | 172   | Caco-2     |
| G>A +6  | 0.358  | 387   | Caco-2     |
| G>A +6  | 0.349  | 241   | liver      |
| insA +7 | 0.976  | 675   | liver      |
| insA +7 | 0.905  | 762   | liver      |
| insA +7 | 0.859  | 575   | liver      |
| insA +7 | 0.856  | 1139  | intestinal |
| insA +7 | 0.699  | 323   | HEK293T    |
| insA +7 | 0.602  | 246   | HEK293T    |
| insA +7 | 0.487  | 344   | HEK293T    |
| insA +7 | 0.438  | 199   | Caco-2     |
| insA +7 | 0.329  | 233   | Caco-2     |
| insA +7 | 0.299  | 324   | Caco-2     |
| C>A +30 | 0.787  | 558   | Caco-2     |
| C>A +30 | 0.768  | 355   | HEK293T    |
| C>A +30 | 0.739  | 622   | liver      |
| C>A +30 | 0.595  | 503   | intestinal |
| 7bp del | 0.21   | 97    | HEK293T    |
| 7bp del | 0.139  | 57    | HEK293T    |
| 7bp del | 0.12   | 85    | HEK293T    |
| 7bp del | 0.09   | 64    | Caco-2     |
| 7bp del | 0.039  | 33    | liver      |

Nicking location of pegRNA      bold Substitution      Insertion      Deletion

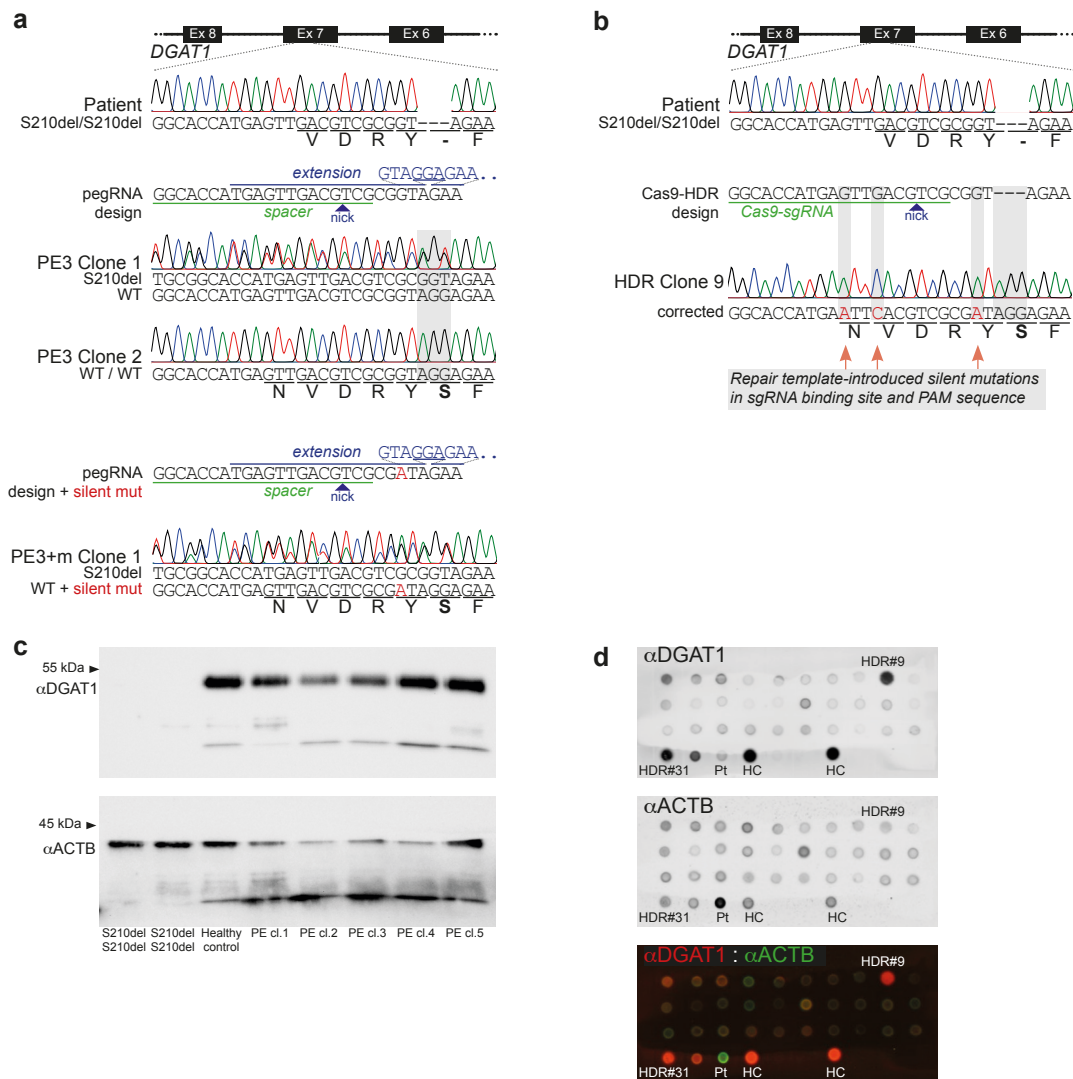
**Supplementary Figure 4**  
**Analysis of editing byproducts after prime editing in HEK293T cells, Caco-2 cells, and liver and intestinal organoid cells**  
Allele frequency tables of the *HEK3* and *CTNNB1* locus after prime editing (PE3) to generate a 5-bp and 6-bp deletion, respectively, directly upstream from the pegRNA nicking position. For each cell type, transfection and high-throughput amplicon sequencing was performed in three replicates. Unwanted byproducts that occurred at a frequency of >0.2% were grouped by pattern to visualize occurrence in each cell type. Note that common unwanted byproduct patterns occur in all cell types, but generally at a very low rate (<1%).



### Supplementary Figure 5

#### Functional correction of *DGAT1*<sup>S210del</sup> by prime editing (PE3)

**a**, Left panel represents outgrowth of single, FACS sorted patient-derived intestinal organoid cells after transfection with GFP-reporter plasmid only or with GFP-reporter plasmid and PE3 plasmids. Right panel represents relative organoid size at day 7 after seeding, which was used as a proxy to quantify proliferation capacity of organoid cells in each condition. Data are represented as mean values  $\pm$  S.D. of three independent experiments. **b**, Representative brightfield images of *DGAT1*<sup>S210del</sup> patient organoids after exposure to 4 mM oleic acid for 24 hours, without (GFP only) and with prime editing (PE3/PE3b). PAM mut stands for conditions in which the PAM-site was mutated by prime editing. White arrowheads indicate surviving organoids. White scale bars are 500  $\mu$ m. PE3+m: PE3 with introduction of PAM mutation. Source data are provided as a Source Data file.

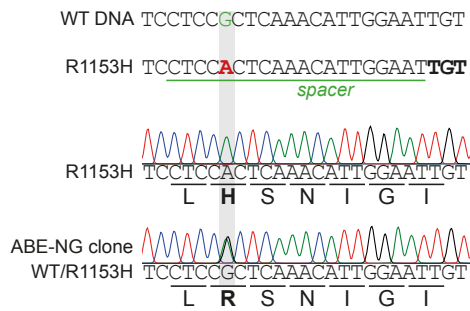


## Supplementary Figure 6

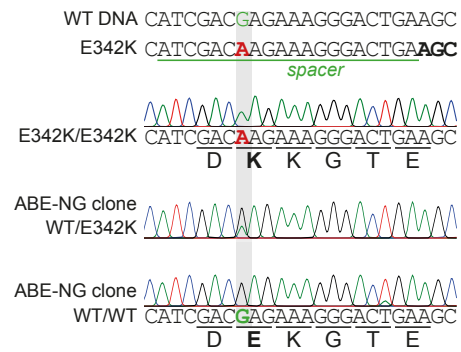
### Prime editing (PE3) and Cas9-mediated HDR to correct *DGAT1*<sup>S210del</sup>

**a**, Design of the pegRNAs used in PE3 to repair the *DGAT1*<sup>S210del</sup> mutation in patient-derived intestinal organoids. Sanger validation of the mutation before editing and after editing in two clonally expanded organoid lines with monoallelic and biallelic repair. Note that PE3+mutation of the PAM results in a silent mutation. **b**, Design of the sgRNA used to repair *DGAT1*<sup>S210del</sup> mutation through Cas9-initiated HDR. A homology template of  $\pm 200$  nucleotides flanking the DSB was used, which contained silent mutations to enhance editing. Sanger validation of the single clone with biallelic repair. **c**, Western blot validation of 5 prime edited (PE) clones. **d**, Dot blot validation of HDR corrected clones; Pt: *DGAT1*<sup>S210del</sup> patient organoids; HC: healthy control organoids; HDR#9: *DGAT1*<sup>S210del</sup> patient organoids after HDR correction, clone #9. Ex: exon, HDR: homology directed repair, Pt: patient, HC: healthy control.

**a** ABCB11 R1153H (G>A) by ABE-NG



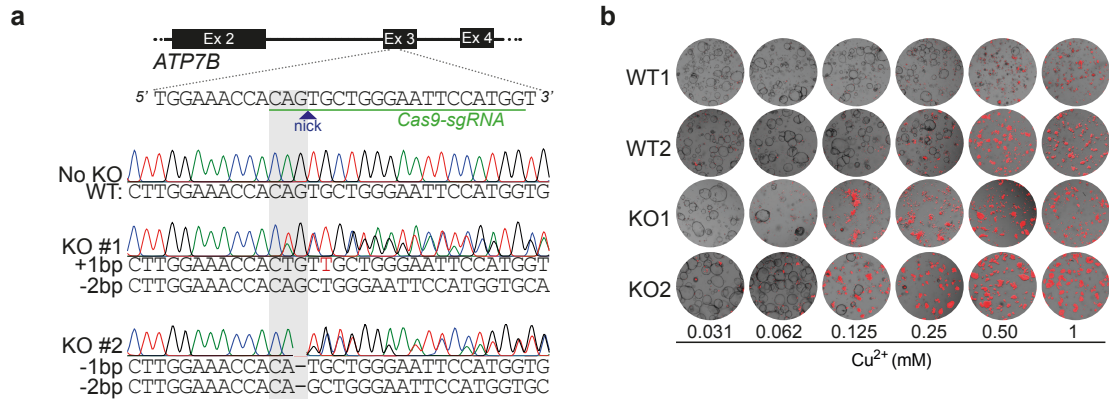
**b** SERPINA1 E342K (G>A) by ABE-NG



**Supplementary Figure 7**

**Correction of *ABCB11*<sup>R1153H</sup> and *SERPINA1*<sup>E342K</sup> by base editing**

**a**, Design of the sgRNA used for adenine base editing (ABE) of the *ABCB11*<sup>R1153H</sup> mutation using ABEmax-NG in liver organoids derived from a patient with BSEP-deficiency. Sanger validation of monoallelic repair by ABE-NG **b**, Design of the sgRNA used for adenine base editing of the *SERPINA1*<sup>E342K</sup> mutation using ABEmax-NG in liver organoids derived from a patient with alpha-1-antitrypsin deficiency. Sanger validation of monoallelic and biallelic repair by ABE-NG. The mutations in **(a)** and **(b)** were also targeted by PE3 but without success; related to Fig. 2g.

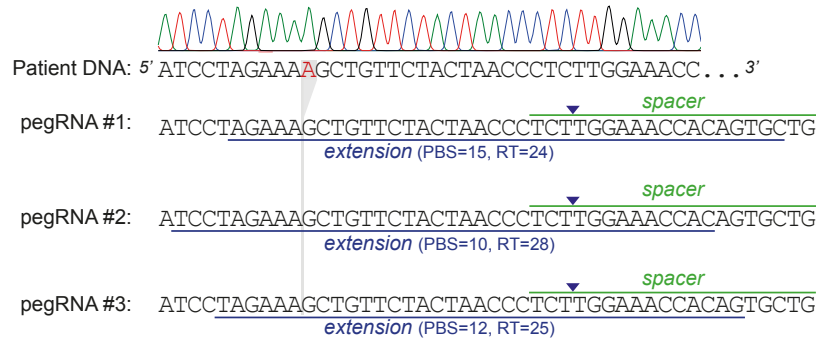


### Supplementary Figure 8

#### Validation of a functional assay for *ATP7B* function

**a**, Design of the sgRNA used to create indels in exon 3 of *ATP7B* in liver organoids, derived from healthy controls, using a cutting Cas9. Sanger validation of two clonal *ATP7B* knock-out (KO) lines, showing heterozygous frameshift mutations. **b**, Propidium iodide staining on wildtype and *ATP7B* knock-out liver organoids after copper exposure. Ex: exon.

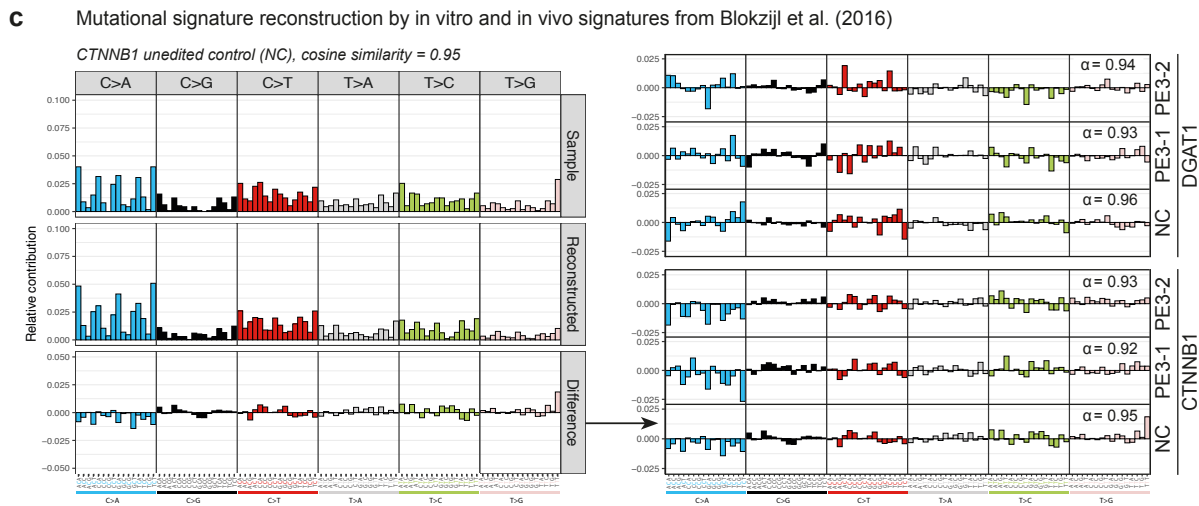
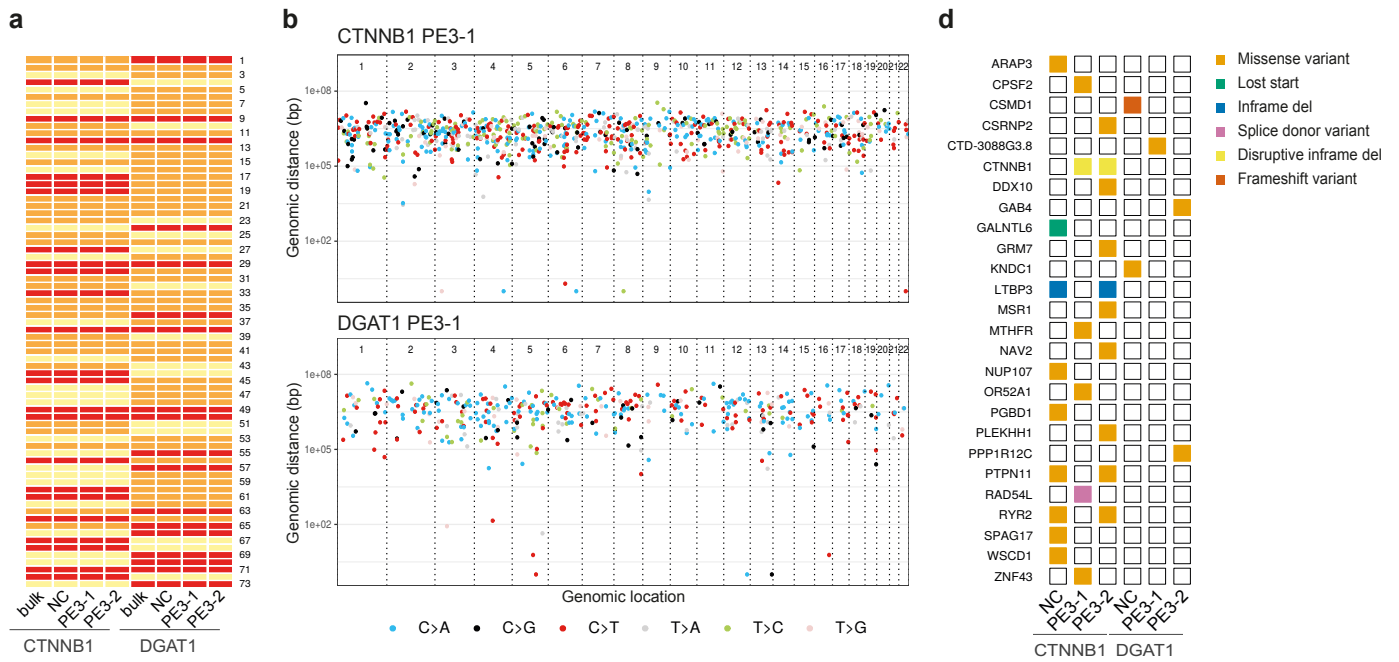
**a**



### Supplementary Figure 9

#### PegRNA designs used for correction of *ATP7B*<sup>S410fs</sup>

Schematic overview of the different pegRNAs used in an effort to correct *ATP7B*<sup>S410fs</sup> mutations in liver organoids derived from a Wilson disease patient. Note that the same spacer was used, but various PBS and RT-template lengths were tested. PBS: primer binding site, RT: reverse transcriptase template.



## Supplementary Figure 10

### Prime editing induces no genome-wide off-target effects

**a**, Fingerprinting of whole-genome-sequenced samples validates shared donor origin of bulk, unedited negative control (NC), and prime edited (PE3-1 and PE3-2) organoid lines. **b**, Rainfall plots of prime edited clonal organoid lines. Every new mutation, relative to the background bulk line, is indicated with a dot and is ordered by genomic location on the X axis. The Y axis shows the genomic distance between each new mutation and the one prior to it (log scale). **c**, Reconstruction of the mutational signature of prime edited and unedited control samples by *in vivo*- and *in vitro*-signatures from Blokzijl et al. (2016). Cosine similarity ( $\alpha$ ) indicates how well the reconstructed signature resembles the sample signature (1= perfect, 0.9= very well, 0= no resemblance). **d**, Overview of new variants in tumor suppressors and oncogenes from COSMIC (Forbes et al., 2017). Note that the disruptive in-frame deletions in *CTNNB1* are the intended, correct mutations of the clonal prime edited *CTNNB1* organoid lines (PE3-1 and PE3-2). Also note that prime edited samples do not acquire more mutations in the considered genes compared to negative control (NC) samples of the same donor line.

**Supplementary Table 1.** Sequences of pegRNAs used in prime editing experiments. All sequences are shown in 5' to 3' orientation. pegRNAs are a concatenation of the spacer sequence, the sgRNA scaffold, and the 3' extension (contains PBS and RT template (RTT)).

| pegRNA                  | spacer sequence      | 3' extension sequence                          | PBS<br>length<br>(nt) | RTT<br>length<br>(nt) | Figure       |
|-------------------------|----------------------|------------------------------------------------|-----------------------|-----------------------|--------------|
| HEK3_del1-5             | GGCCCAGACTGAGCACGTGA | TGGAGGAAGCAGGGCTTCCTTTCTCTGCCGTGCTCAGTCTG      | 13                    | 29                    | 1b,h, S4     |
| HEK3_26CtoG             | GGCCCAGACTGAGCACGTGA | TGGAGGAACCAGGGCTTCCTTTCTCTGCCATCACGTGCTCAGTCTG | 13                    | 34                    | 1c           |
| CTNNB1_S1E1_del1-6      | CAACAGTCTTACCTGGACTC | TGGCACCAGAATGGATTTCCAGGTAAGAC                  | 12                    | 17                    | 1d,e,g,h, S4 |
| CTNNB1_S1E2_del3-5      | CAACAGTCTTACCTGGACTC | ACCAGAATGGATTTCAGTCCAGGTAAGACTGT               | 15                    | 16                    | 1d,e,g       |
| CTNNB1_S2E1_del4-12     | CTGGCAGCAACAGTCTTACC | TGGTAGTGGCACCAGAATGGTAAGACTGTTGCTGCC           | 15                    | 21                    | 1d,e,g       |
| CTNNB1_S2E2_del4-18     | CTGGCAGCAACAGTCTTACC | TGGCACCAGAATGGATTCGGTAAGACTGTTGCT              | 12                    | 21                    | 1d,e,g       |
| ABCB11-D482G-create     | CCATGCTCTCTAGGTGACCG | AATGTCATGGCCACCTACGGTCACCTAGAGAGC              | 13                    | 20                    | S3           |
| DGAT1_ins9-11_GGA_S1E1  | GGCACCATGAGTTGACGTCG | AAGCTCTTCTCCTACCGCGACGTCAACTCA                 | 10                    | 20                    | 2b,c,d, S5   |
| DGAT1_ins9-11_GGA_S1E2  | GGCACCATGAGTTGACGTCG | AAGCTCTTCTCCTATCGCGACGTCAACTCA                 | 10                    | 20                    | 2d, S5       |
| ATP7B-del19-pegRNA1     | CAGCACTGTGGTTTCCAAGA | AGAAAGCTGTTCTACTAATCCTCTTGGAACCACAGTGC         | 15                    | 24                    | 2k, S9       |
| ATP7B-del19-pegRNA2     | CAGCACTGTGGTTTCCAAGA | TCCTAGAAAGCTGTTCTACTAATCCTCTTGGAACCAC          | 10                    | 28                    | 2k, S9       |
| ATP7B-del19-pegRNA3     | CAGCACTGTGGTTTCCAAGA | TAGAAAGCTGTTCTACTAATCCTCTTGGAACCACAG           | 12                    | 25                    | 2k, S9       |
| ABCB11-R1153H-correct-1 | AAACAATTCCAATGTTTGAG | CCAGTTCCTCCGCTCAAACATTGGAATTGT                 | 12                    | 18                    | 2g, S7       |
| ABCB11-R1153H-correct-2 | CAATTCCAATGTTTGAGTGG | TGTCCAGTTCCTCCGCTCAAACATTGGAAT                 | 12                    | 18                    | 2g, S7       |
| SERPINA1-E342K-correct  | TCCCTCCAGGCCGTGCATA  | TCCCTTTCTCGTCGATGGTCAGCACAGCCTTATGCACGGCCTGGAG | 12                    | 34                    | 2g, S7       |

**Supplementary Table 2.** Sequences of nicking sgRNAs used in prime editing (PE3), HDR, Cas9-cutting, and base editing experiments. Sequence of HDR template used for HDR in *DGAT1* .

| nickng sgRNA (PE3)       | spacer sequence                                                                                                                                                                                              | Figure       |
|--------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------|
| HEK3+90                  | GTCAACCAGTATCCCGGTGC                                                                                                                                                                                         | 1b,c,h, S4   |
| CTNNB1+86/+93            | CCACTCATACAGGACTTGGG                                                                                                                                                                                         | 1d,e,g,h, S4 |
| ABCB11_482+80            | ATGGTGGTAGAGAACAGAAC                                                                                                                                                                                         | S3           |
| DGAT1+46                 | GGGTCCCCTGCTGGCGCTGA                                                                                                                                                                                         | 2b,c,d, S5   |
| DGAT1_PE3b               | CCTACCGCGACGTCAACTCA                                                                                                                                                                                         | 2d, S5       |
| DGAT1_PE3b+PAMmut        | CCTATCGCGACGTCAACTCA                                                                                                                                                                                         | 2d, S5       |
| ATP7B_PE3b               | AGCTGTTCTACTAATCCTCT                                                                                                                                                                                         | 2k, S9       |
| ATP7B-420-39             | TCCATGGTGCAAACACTACAGA                                                                                                                                                                                       | 2k, S9       |
| ABCB11-R1153H+45/+42     | CTCTATTGGCAGATGATAGA                                                                                                                                                                                         | 2g, S7       |
| SERPINA1-E342K+54        | GGGTATGGCCTCTAAAAACA                                                                                                                                                                                         | 2g, S7       |
| base edit sgRNA (ABE-NG) | spacer sequence                                                                                                                                                                                              | Figure       |
| ABCB11-R1153H            | CTCCACTCAAACATTGGAAT                                                                                                                                                                                         | 2g, S7       |
| SERPINA1-E342K           | ATCGACAAGAAAGGGACTGA                                                                                                                                                                                         | 2g, S7       |
| HDR sgRNA                | spacer sequence                                                                                                                                                                                              | Figure       |
| DGAT1_HDR_sgRNA          | GGCACCATGAGTTGACGTCG                                                                                                                                                                                         | 2f, S6       |
| HDR repair template      | HDR template sequence                                                                                                                                                                                        |              |
| DGAT1_HDR_template       | GCTGTGGAGGCCTGAGTCCACCTCTCCTGCAGTGGGCTCCCTGCTGGCGCTGATGGCGCACACCATCCTCTTCCT<br>CAAGCTCTTCTCCTATCGCGACGTGAATTTCATGGTGTCGCAGAGCCAGGGCCAAGGCTGGTGAGGGGCTGCCAGG<br>GGCTGGGGCTGCCTGCTGGGGGGCTGGGCAGCAGCAGGGCCCCAC |              |
| Cas9-cutting sgRNA       | spacer sequence                                                                                                                                                                                              | Figure       |
| ATP7B_exon3-cas9-KO      | CCATGGAATTCCCAGCACTG                                                                                                                                                                                         | S8           |

**Supplementary Table 3.** Sequences of PCR and sequencing primers used for amplification and sequencing of target regions. All sequences are shown in 5' to 3' orientation.

| PCR and Seq primers | primer sequence                                                 | Figure     |
|---------------------|-----------------------------------------------------------------|------------|
| HEK3_PCR-Fw         | ATGTGGGCTGCCTAGAAAGG                                            | 1b,c       |
| HEK3_PCR-Rv         | GGTGCTGAAAGCCACTGGGC                                            | 1b,c       |
| HEK3_Seq-Fw         | GGGAAACGCCCATGCAATTAG                                           | 1b,c       |
| CTNNB1-ex3-PCR-Fw   | AGCAGGAATCTAGTCTGGATGA                                          | 1f         |
| CTNNB1-ex3-PCR-Rv   | ACTCACTATCCACAGTTCAGCA                                          | 1f         |
| CTNNB1-ex3-Seq-Fw   | GGATGCAGTACCATTCTTCCAC                                          | 1f         |
| ABCB11_482-PCR-Fw   | CCACAATGAATTGTTCTGCAGC                                          | S3         |
| ABCB11-482_PCR-Rv   | CCTTCTATGACCTCTTAGTTTCTCC                                       | S3         |
| ABCB11-482_Seq-Fw   | CCACATTTCAAGAGGAATTGTTTCC                                       | S3         |
| DGAT1_PCR-Fw        | ATGCCTGGTTATTGGTGAGC                                            | 2b, S6     |
| DGAT1_PCR-Rv        | TGAGCTCGTAGCACAAGGTG                                            | 2b, S6     |
| DGAT1_Seq-Fw        | GCATTCCAGGTTGAGAAGCG                                            | 2b, S6     |
| ATP7B_PCR-Fw        | CCCCGCATGCAGTAAGTATT                                            | 2h, S8, S9 |
| ATP7B_PCR-Rv        | AACACAATGCCAGTTATACAAGGA                                        | 2i, S7, S8 |
| ATP7B-Seq-Fw        | AGGTGAAGTTACGGGGTAGC                                            | 2i, S7, S8 |
| ABCB11_1153-PCR-Fw  | CTGTAGAGCCAGAGTTCAGG                                            | 2g, S7     |
| ABCB11_1153-PCR-Rv  | CATTCTACTTCTCCCCATCC                                            | 2g, S7     |
| SERPINA1_342-PCR-Fw | GTCAGGAAGATGGACAGAGG                                            | 2g, S7     |
| SERPINA1_342-PCR-Rv | GGCAGTTATTTTGGGTGGG                                             | 2g, S7     |
| HEK3_HTS-Fw         | ACACTCTTCCCTACACGACGCTCTTCCGATCTNNNNATGTGGGCTGCCTAGAAAGG        | 1h         |
| HEK3_HTS-Rv         | TGGAGTTCAGACGTGTGCTCTTCCGATCTCCAGCCAAACTTGTC AACC               | 1h         |
| CTNNB1_HTS-Fw       | ACACTCTTCCCTACACGACGCTCTTCCGATCTNNNNCAATCTACTAATGCTAATACTGTTTCG | 1h         |
| CTNNB1_HTS-Rv       | TGGAGTTCAGACGTGTGCTCTTCCGATCTAAGGACTGAGAAAATCCCTGTTC            | 1h         |